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The final deprotection step in oligonucleotide synthesis is reduced to a mild and rapid ammonia treatment by using labile base-protecting groups

J.C.Schulhof, D.Molko and R.Teoule

Laboratoires de Chimie, Departement de Recherche Fondamentale, Centre d'Etudes Nucleaires de Grenoble, 85 X, F-38041 Grenoble Cedex, France

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ABSTRACT:

Phenoxyacetyl (pac) and methoxyacetyl (mac) for adenine and guanine, isobutyryl for cytosine, were successfully applied as amino protecting groups both in phosphotriester and phosphoramidite approaches. As shown by N.M.R. and H.P.L.C. analysis, they are completely deblocked in less than four hours in 29% ammonia at room temperature allowing the preparation of modified DNA containing alkali labile bases such as saturated pyrimidines. The stability of N6-phenoxyacetyl-deoxyadenosine versus depurination in acidic conditions used in the detritylation step was favorably compared with that of the classic N6-benzoyl protected adenine.

INTRODUCTION:

The choice of isobutyryl and benzoyl for the protection of the exocyclic amino functions of guanine, adenine and cytosine in oligonucleotide synthesis dates back to the work of Khorana and co-authors with the phosphodiester methodology (1,2). With respect to the progress achieved in the field of phosphotriester and phosphoramidite synthesis, these protecting groups present now a few shortcomings :

Once they are grafted on the nucleic bases, their stability is such that they require a long and drastic deprotection step: (17 hours in concentrated ammonia at 60°C, in sealed vials) ; in this way, the deprotection step has become the time-consuming part in the synthesis of DNA fragments and is opposed to their immediate use. In the methods presently available, the assembly of a 30-mer takes less than 6 hours and the conditions of condensation are much milder than in the phosphodiester approach. Therefore, such high stability is no more necessary.

Furthermore, the usual ammonia overnight treatment necessary to hydrolyse the classically used benzoyl and isobutyryl

groups forbids the synthesis of modified oligonucleotides containing alkali-sensitive bases.

The deprotection time required by these classical groups, at the end of the oligomer synthesis, is not homogenous. In a comrehensive study, Köster et al (3) measured their respective values. N-deacylation in sodium hydroxide/methanol appears to be thirty times longer for N2-isobutyryl-2'-deoxyguanosine than for N4-benzoyl-2'-deoxycytidine. We can also note that the usual treatment (at least 17 hours in concentrated ammonia at 60°C) is not quite sufficient to remove totally the isobutyryl groups from deoxyguanosine residue, as shown by high field 1H-NMR (4 and published spectra).

In order to suppress these drawbacks, many studies have been carried out in search of new protecting groups. Various substitutions of the benzoyl (3) and butyryl groups (5) have been suggested. Of interest are the p.t.butylphenoxyacetyl, p.t.butylphenylacetyl (3,6), phenylacetyl (7), the benzyloxycarbonyl (CBZ) (8) and (9-fluoroenyl)-methoxycarbonyl (FMOC) (9) groups : These five groups exhibit an important lability, far below the classically used benzoyl and isobutyryl groups. Besides, we can also mention among other compounds : the 2-nitrophenylsulfenyl (NPS) (10), isovaleryl, pivaloyl, propionyl (5,11), 2-methylpropanoyl (12), nitrophenylethoxycarbonyl (13), dialkylformamidines (14), succinyl, phthaloyl and substitued (15), and more recently (3-methoxy 4-phenoxy)benzoyl (16).

From these papers, it appears that :

-Only very few of these protecting groups have actually been tested in oligonucleotide synthesis; and furthermore, have simply been used to build the protected nucleotide units.

-Many groups show very high stability.

-Many of them require a supplementary deprotection step in addition to the ammonia treatment, and this is not desirable to render the oligonucleotide synthesis practical and rapid.

-Often, the protecting groups proposed concern only one nucleic base whereas a set of compatible groups for adenine, guanine and cytosine would be of greater interest.

-The selected groups are rarely commercially available.

-Yields have to be taken into account when new groups are to

be used in oligonucleotide synthesis.

In this work, we wish to propose a set of efficient groups for the protection of the exocyclic amino functions of nucleic bases. These groups should allow a gain of time in the preparation of DNA fragments as well as the insertion of modified labile bases in the chain. They are stable enough during the assembling steps and removed totally from the oligomer after synthesis within four hours in 29% ammonia at room temperature.

RESULTS AND DISCUSSION:

1)-SYNTHESIS OF N-PROTECTED NUCLEOSIDES:

Various acyl groups were introduced on the exocyclic amino functions of adenine, guanine and cytosine : Methoxyacetyl, phenoxyacetyl, 2-chlorophenoxyacetyl were grafted on deoxyguanosine, phenoxyacetyl on deoxyadenosine, and isobutyryl on deoxycytidine. Then their respective deprotection times were evaluated in 29% ammonia/pyridine (80/20) at room temperature and compared to those of the classically protected nucleosides. The kinetics were followed by thin layer chromatography : samples were taken up at different reaction times and immediately neutralized by dilute acetic acid. The results are summarized in the following table :

Table 1 : Comparison of the N-deacylation time required by the protecting groups by t.l.c. measurements in 29%ammonia/pyridine (80/20) at 20°C.

Compound :	Half time of N-deacylation in 20°C 29% ammonia (t 1/2).
N2-isobutyryl-2'-deoxyguanosine	10 hours .
N6-benzoyl-2'-deoxyadenosine	8 hours .
N4-benzoyl-2'-deoxycytidine	2 hours .
1. N4-isobutyryl-2'-deoxycytidine	30 minutes .
2. N6-phenoxyacetyl-2'-deoxyadenosine	7 minutes .
3. N2-phenoxyacetyl-2'-deoxyguanosine	15 minutes .
4. N2-methoxyacetyl-2'-deoxyguanosine	45 minutes .
N2-chlorophenoxy-acetyl-2'-deoxyguanosine	8 minutes .

These results confirm the large differences between the classical amino protections (3). They show for the various protecting groups proposed, half-life times of deprotection from 7 to 45 minutes. Hence, these groups exhibit correct values to expect a final removal from oligonucleotides within a few hours : final deprotection of such protected oligonucleotides should take at room temperature from 4 to 6 hours (t 1/2 x 8), depending on which group is used to protect the guanine residue. However, it is worth noting that the deprotection time of the free nucleoside may be quite different from that of the nucleoside inserted in the DNA chain.

Yield of synthesis: Different pathways were studied in order to improve the yields of synthesis.

-The efficient transient protection (17) was successfully applied for deoxycytidine, allowing the synthesis of N4-isobutyryl-2'-deoxycytidine with a 60% yield. This method loses its efficiency with the phenoxyacetyl group, presumably because phenoxyacetyl chloride is too drastic and provokes degradation of nucleosides and the formation of colored side-products.

-Khorana's classical procedure was employed to protect the adenine moiety. In this case, anhydrid can be used and N6-phenoxy acetyl-2'-deoxyadenosine was obtained with a 65% yield. It is worth noting that the intermediate hydrolysis of the two hydroxyl groups of the sugar moiety can be carried out by a mixture of triethylamine-pyridine-water (20/20/60) instead of sodium hydroxyde and neutralization with a Dowex resin.

-For deoxyguanosine, the procedure introduced by Benseler et al (18) gave excellent results. This consists in mixing 1-hydroxy benzotriazole and the acid chloride to get a milder acylating agent. Thus, the labile phenoxyacetyl group was introduced on the amino function with a 80% yield, whereas it was only of 60% with Khorana's classical procedure.

-Methoxyacetyl chloride gave good yield, but the methoxyacetyl group is less labile than the phenoxyacetyl one.

Considering facility of synthesis, yield and deprotection time, methoxyacetyl and phenoxyacetyl were selected for deoxyguanosine, phenoxyacetyl for deoxyadenosine, and isobutyryl for

deoxycytidine. The identity and purity of these protected nucleosides 1-4 were checked by mass spectroscopy and by high field 1H-NMR. Data of the compounds 1-4 are reported in the experimental part.

The stability of these compounds towards potential alkaline reagents used in oligonucleotide synthesis was checked. Thus, interaction with lutidine, methyl imidazole, dimethylamino pyridine, was tested in various oligonucleotide synthesis conditions and showed no deprotection of the base.

2)-COMPARATIVE STUDY OF DEPURINATION:

Protic acid conditions, used to remove the dimethoxytrityl group, is known to provoke, in some extent, the breakage of the N-glycosylic bond of purine nucleosides. This lowers the yield and the integrity of the chain. Deoxyadenosine is more sensitive than deoxyguanosine and its depurination appears more or less rapidly in acidic conditions, depending on the nature of the protecting group grafted on the amino function. Indeed, acylation of this function eases the protonation of the N7 and thereby, the hydrolysis under acidic conditions. The introduction of a bulkier group is supposed to inhibit the rotation around the N-glycosyl bond and thus to improve the resistivity of the derivative (19,20).

In order to be suitable for oligonucleotide synthesis, a protecting group must not destabilize the glycosylic bond. We have therefore measured and compared the depurination rates of N6-phenoxyacetyl-2'-deoxyadenosine and N6-benzoyl-2'-deoxyadenosine in 80% acetic acid. The hydrolysis of these two compounds was carried in the same flask and the kinetic was followed by H.P.L.C. with a reverse phase column. Figure 1-a shows chromatograms before hydrolysis (t=0), after 15 minutes and after 2 hours of reaction. In figure 1-b, concentration is plotted versus reaction time. Analysis of the different curves give, in these experimental conditions, respective half-depurination times of 80 and 100 minutes for the benzoyl and phenoxyacetyl derivatives. These values show an increase in stability towards depurination of approximately 20% in favor of the phenoxyacetyl group.

3)-PREPARATION OF THE MONOMERS FOR DNA FRAGMENTS ASSEMBLY:

The acid labile 4,4'-dimethoxytrityl group was used to pro-

Figure 1-a:

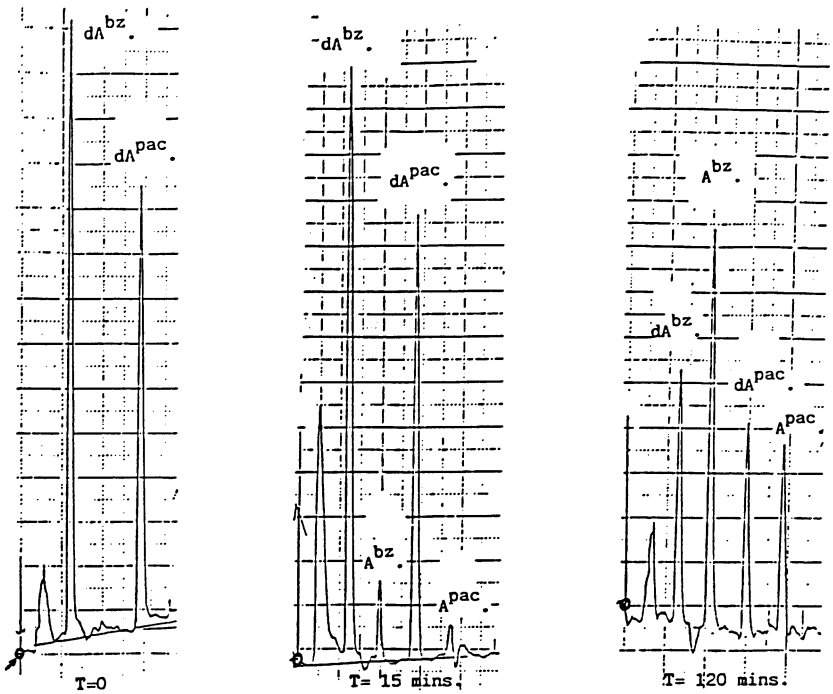


Figure 1-b:

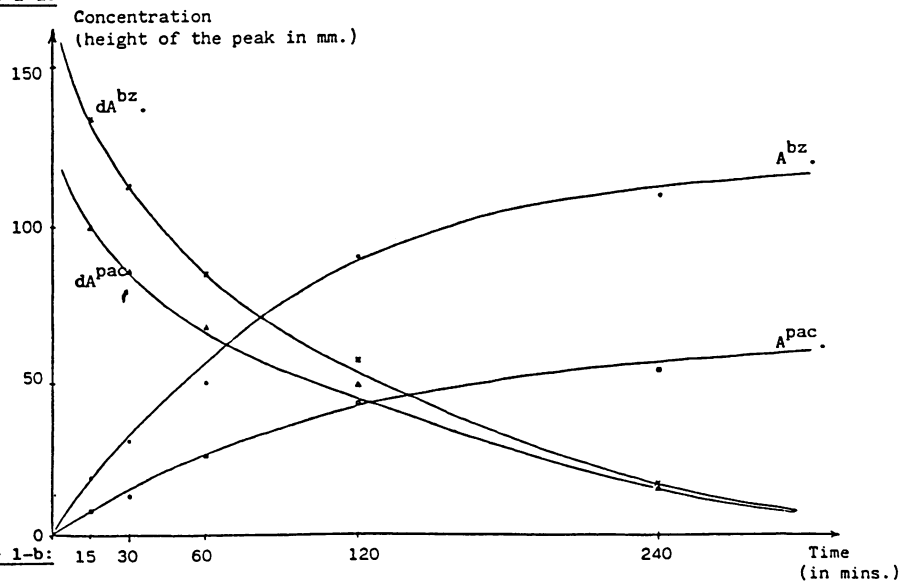
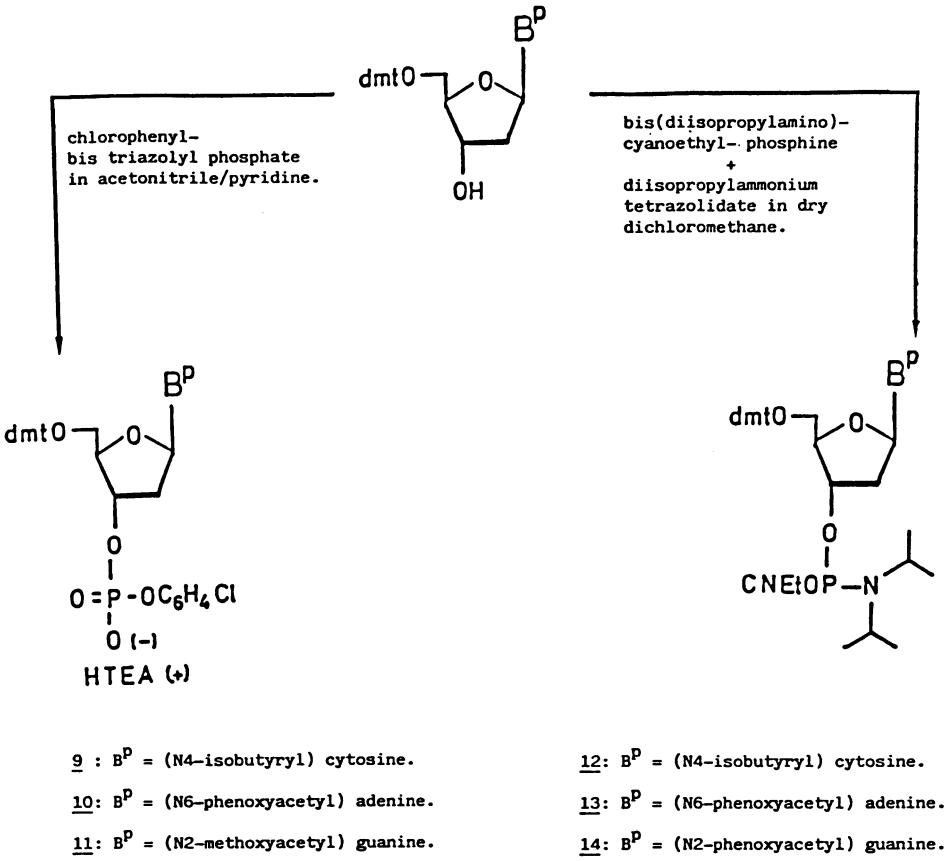


Figure 1: Comparative study of the deoxyadenosines depurination.
1-a: H.P.L.C. spectra of the depurination in acidic conditions at different reaction times: T = 0 , 15 , and 120 minutes.
1-b: Evolution of the species. (A^{bz}:N6-benzoyl-adenine ; A^{pac}: N6-phenoxyacetyl-adenine).



9 : B^P = (N4-isobutyryl) cytosine.

10: B^P = (N6-phenoxyacetyl) adenine.

11: B^P = (N2-methoxyacetyl) guanine.

12: B^P = (N4-isobutyryl) cytosine.

13: B^P = (N6-phenoxyacetyl) adenine.

14: B^P = (N2-phenoxyacetyl) guanine.

Figure 2 : Preparation of phosphotriester and phosphoramidite derivatives starting from the newly N-protected dimethoxytritylated nucleosides.

tect the 5' hydroxy function of the sugar moiety. After purification by column chromatography, the expected compounds 5-8 were checked by mass spectroscopy and 1H-NMR.

The nucleosides were then phosphorylated. According to the procedures reported in figure 2, both phosphotriester and phosphoramidite derivatives were prepared.

-Phosphotriester approach: The monomers needed for the solid phase phosphotriester method were obtained by reaction of the protected nucleosides with a mixture of 2-chlorophenyl-dichlorophosphate, triazole and triethylamine (21,22). After hydrolysis and column chromatography on silica gel, derivatives 9-11 were

checked by 1H-NMR, 31-NMR, and mass spectroscopy.

-Phosphoramidite approach: The following 5'-tritylated compounds (5-7) were treated by cyanoethyl-bisdiisopropylamino-phosphine in the presence of diisopropylammonium tetrazolidate (23,24). The phosphoramidite monomers were purified on a Merck Lobar silica gel column with a chloroform/triethylamine/hexane elution mixture and were stored under argon as dry white powders. Homogeneity of the nucleotidic monomers 12-14 was checked by 31P-NMR, by 1H-NMR and mass spectroscopy. Data of the nucleotidic blocks are summarized in the experimental section.

4)-SYNTHESIS OF OLIGODEOXYRIBONUCLEOTIDES:

-Phosphotriester approach:

The triethylammonium salts of phosphotriester monomers 9-11 and the corresponding derivatives of thymidine and deoxyuridine were used to synthesize pentadecamers and heptadecamers containing the uracil base at various positions. Efimov's procedure (21,22) was used, and the controlled pore glass (CPG) grafted with the oligonucleotide was treated with 29% ammonia at room temperature for six hours (8 x half-deprotection time of N2-methoxyacetyl-deoxyguanosine). The crude material was desalted by size exclusion gel chromatography on Sephadex and 5'-end labelled by 32P ATP and T4 polynucleotide kinase. The sequence was confirmed by the Maxam and Gilbert procedure (25,26). Oligonucleotides were purified by polyacrylamide gel electrophoresis by cutting the desired lengths and, after elution of the DNA fragments, were further purified by H.P.L.C.

-Phosphoramidite approach:

*Study of the base deprotection in a synthesized tetramer: The phosphoramidite solid phase method is at the moment the most widely used. A tetramer TGAC was first prepared by the phosphoramidite solid phase method with compounds 12-14 as starting material, in order to check that all the base-protecting groups are removed after oligonucleotide synthesis within 4 hours of ammonia treatment at room temperature. Indeed, with phenoxyacetyl as the protecting group of guanine, 4 hours of treatment should be sufficient in order to deprotect the synthesized chains (4 hours=8 x half-deprotection time of isobutyryl-deoxycytidine). In this purpose, a large amount of tetramer (=10 mg) was prepared

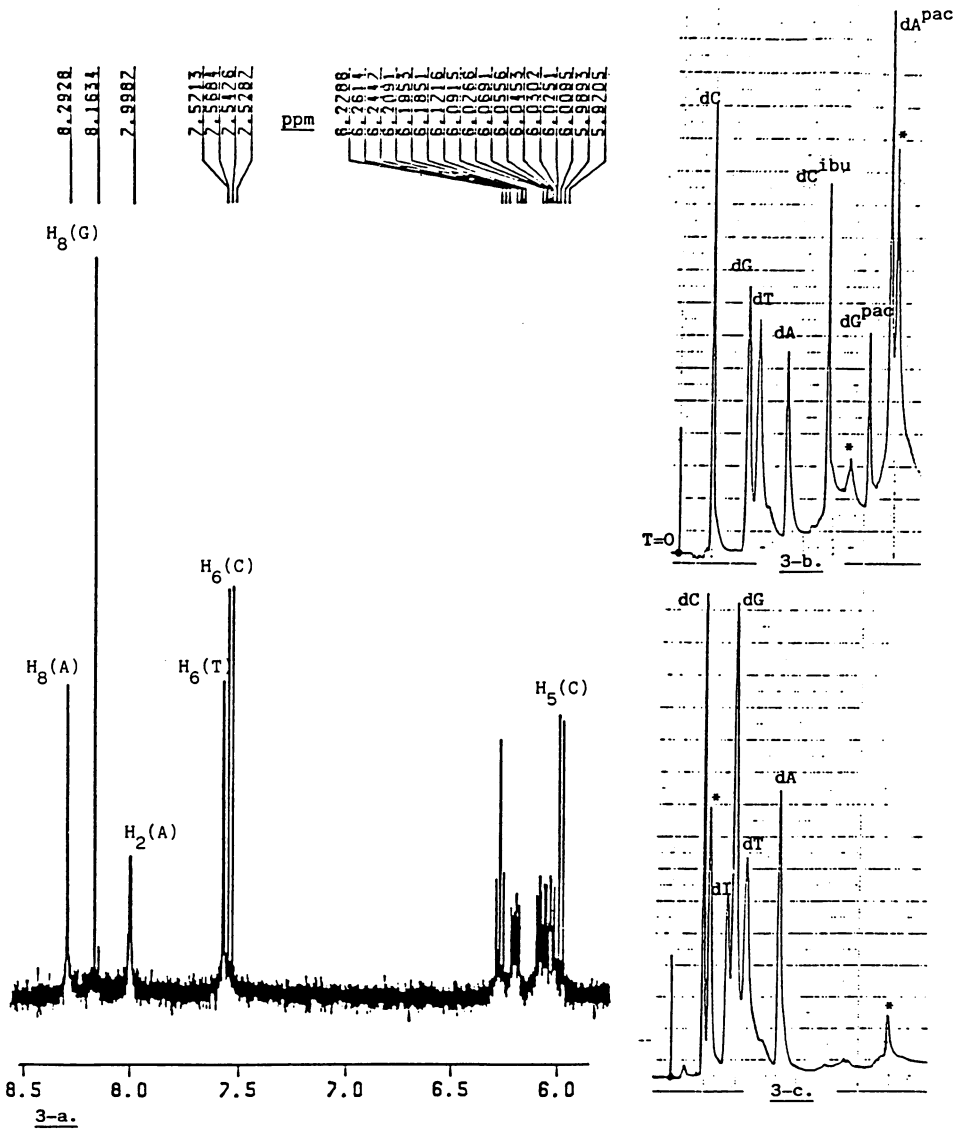


Figure 3: Deprotection of the tetramer d(CpApGpT) by a treatment of 4 hours of 29% ammonia at 20°C.

3-a: N.M.R. spectrum (nucleic bases, anomeric and aromatic part of the spectrum) showing that no protecting groups are left on the tetramer.

3-b: H.P.L.C. spectrum of a mixture of N-protected and unprotected nucleosides.

3-c: After enzymatic digestion of the tetramer, H.P.L.C. confirm that no protected monomers are left. (* : these peaks belong to the blank).

and the crude support containing this protected tetramer was divided into three equal parts. The time necessary to release oligomers from the CPG support has been measured and is of two hours. So the three parts were treated by 29% ammonia at 20°C for respectively 2, 4, and 8 hours and were purified by using a size-exclusion gel high performance liquid chromatography "I-125 Protein Analysis" column (Waters) (27), with 5 mM triethylammonium acetate,pH=6.6 as the eluent. This size separating chromatography does not eliminate unwanted partially-protected tetramer, so NMR will reveal the base-protecting groups if they are present. After evaporation and several lyophilizations with deuterated water to eliminate the volatile buffer, the three different portions were studied by high field 1H-NMR (Brüker 400 MHz). Analysis showed that no amino-protecting groups were left in none of the 2, 4, or 8 hours alkali-treated samples. A NMR spectrum of the 2 hours depro tected tetramer is represented in figure 3-a : the peaks corresponding to the four anomeric protons of the four sugar moieties appear without any contamination. Furthermore, any rests of phenoxyacetyl would give supplementary lines visible in this aromatic region of the spectrum. Rests of isobutyryl on deoxycytidine provide large shifts of the H5 and H6 peaks which are not observed here. So, after two hours of treatment in 29% ammonia, no amino protecting groups are left on the different bases of the tetramer.

To confirm these results, the three lots of tetramers (2,4,8 hours) were enzymatically treated by a mixture of snake venom phosphodiesterase and by alkaline phosphatase. The monomeric nucleosides obtained in this way were then compared, in defined chromatographic conditions, to synthetic pure nucleosides with and without protection on the base. This analysis was carried out on a C-18 reverse phase column. Here again, in the limits of sensitivity of the methodology, it is not possible to detect any protected nucleosides in the mixture, as it is shown by comparison of figure 3-b and 3-c.

*Synthesis of oligonucleotides: Taking into account the results obtained with the tetramer described above, synthesis of longer oligonucleotides was then undertaken. An ammonia treatment of 2 hours may be sufficient but, for the synthesis of longer

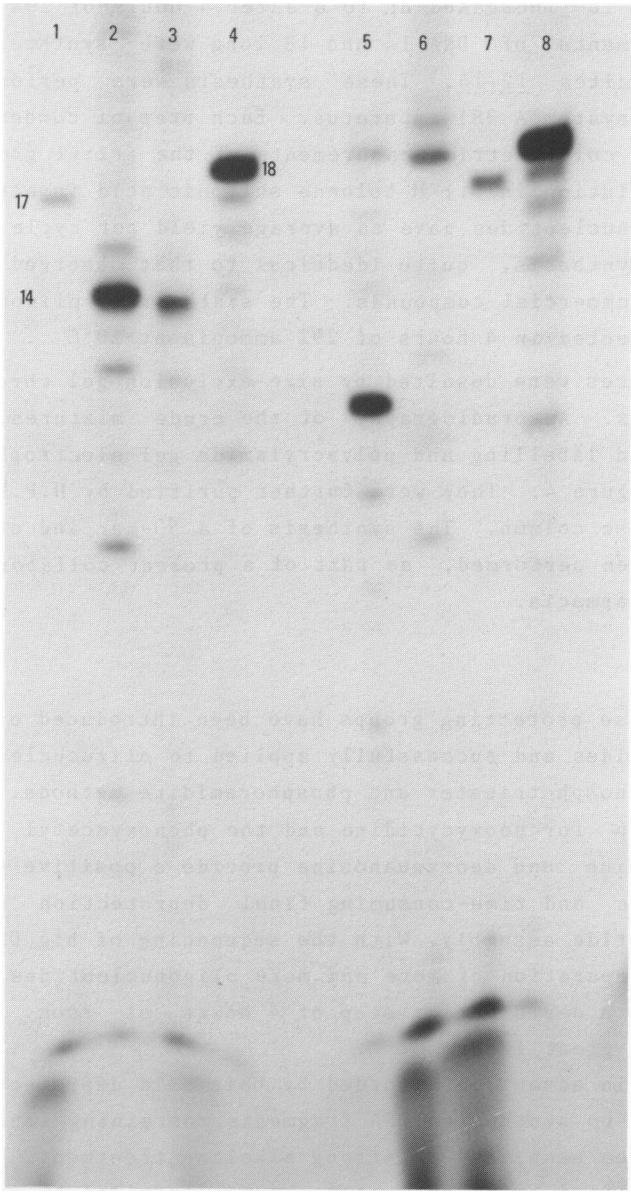


Figure 4: Autoradiography of the oligonucleotides crude mixtures obtained from the cyanoethyl-bis diisopropylamino-phosphoramidite derivatives.
Lanes 1,7: 17-mer of reference ; Lanes 2,3: 14-mers ; Lanes 4,6,8: 18-mers ; Lane 5: 11-mer.

fragments, is increased up to a safer 4 hours of 29% ammonia at 20°C. Fragments of DNA 14 and 18 long were synthesized using phosphoramidites 12-14. These synthesis were performed on a Applied Biosystem A 381 apparatus. Each step of condensation was checked by colorimetric measurements of the trityl group released, by dilution in 0.1 M toluene sulfonic acid in acetonitrile. These new nucleotides gave an average yield per cycle of 98-99% in each synthesis, quite identical to that observed with the classical commercial compounds. The synthesized oligonucleotides were deprotected in 4 hours of 29% ammonia at 20°C . Then, the crude mixtures were desalted by size-exclusion gel chromatography on Sephadex. Autoradiography of the crude mixtures, obtained after 5'-end labelling and polyacrylamide gel electrophoresis, is given in Figure 4. They were further purified by H.P.L.C. with a reverse phase column. The synthesis of a 50-mer and of a 70-mer has also been performed, as part of a present collaboration with the firm Pharmacia.

CONCLUSION:

New base protecting groups have been introduced onto protected nucleotides and successfully applied to oligonucleotide synthesis by phosphotriester and phosphoramidite methods. The isobutyryl group for deoxycytidine and the phenoxyacetyl group for deoxyadenosine and deoxyguanosine provide a positive solution to the drastic and time-consuming final deprotection step after oligonucleotide assembly. With the sequencing of big DNAs, requiring the preparation of more and more oligonucleotides, the time gained by a deprotection step of 4 hours at room temperature could be of great interest.

The main advantage afforded by this mild deprotection is the possibility to synthesize DNA fragments containing labile heterocyclic bases sensitive to strong alkaline treatment. Incorporation of modified bases such as compounds provided by gamma radiolysis is presently studied. 5,6-dihydrothymidine, whose stability is compatible with a short ammonia treatment at room temperature, will be the first incorporated.

EXPERIMENTAL SECTION:

2'-deoxynucleosides were purchased from Cruachan Chemical Co., phenoxyacetyl chloride and methoxyacetyl chloride from Janssen. The solvents were distilled before use. Enzymes came from Boehringer-Mannheim; acetonitrile used for oligonucleotide synthesis was purchased from Rathburn. NMR spectroscopy was performed on Bruker WM 250 and Bruker AM 400. The chemical shifts in 31P-NMR spectra are given in ppm relative to 85% H3PO4 as an external standard and 1H-NMR spectra in ppm relative to TMS as an internal standard. Mass spectrometry was performed on Kratos MS 50 for positive(pos.) or negative(neg.) ion FAB-MS and pyrolysis-MS.

Preparation of N4-isobutyryl-2'-deoxycytidine : (compound 1)

2'-deoxycytidine (4 mmoles; 910 mg) was dried by two evaporations with dry pyridine (10 ml), then dissolved in 15 ml of dry pyridine. Trimethylchlorosilane (20 mmoles; 2.1 ml) was added dropwise and the reaction was left for 20 minutes. Isobutyryl chloride (16 mmoles; 1.65 ml) was added and acylation was complete after 2 hours at room temperature. The mixture cooled at 0 °C was submitted to 3 ml of water and after 5 minutes, to 3 ml of 29% ammonia. This solution was then evaporated to near dryness and dissolved in 75 ml of water to be washed by 3 x 50 ml of diethyl ether. By concentrating the aqueous phase, N4-isobutyryl-2'-deoxycytidine precipitated as a white compound. The solution was kept overnight at 5 °C and filtration gave the desired product with a yield of 60% (720 mg). Rf=0.55 in chloroform-methanol 80/20 (v/v).

1H-NMR (CD3OD): 7.50&8.53(d,H5,H6); 6.25(t,H1'); 4.4(m,H3'); 4.02(m,H4'); 3.7-3.9(m,2H,H5'H5''); 2.7(m,H ibu); 2.15-2.6(m,2H, H2',H2''); 1.21(m,6H,2(CH3) ibu). FAB-MS (PEG matrix, pos.ions): molecular peak +H /e: 298-11% ; N-protected base +H /e: 182-100%.

Preparation of N6-phenoxyacetyl-2'-deoxyadenosine: (compound 2)

2'-deoxyadenosine (4 mmoles; 1025 mg) was dried by two evaporation with dry pyridine (10 ml). It was then dissolved in 20 ml of dry pyridine and treated with phenoxyacetic anhydride (24 mmoles; 7.2 g). After 90 minutes of stirring at room temperature, the reaction was checked for completion by tlc. Water (3 ml) was added to destroy the excess of anhydride and this reaction mix-

ture was diluted to 75 ml by chloroform. The phase was extracted with 3 x 50 ml of 5% NaHCO3 in water followed by 50 ml of water. The organic solution was evaporated to a yellowish gum. A mixture of triethylamine-pyridine-water 20/20/60 was poured over it to deprotect the 3'and 5'-hydroxy functions. After 15 minutes at room temperature, the solution was evaporated to dryness. The N6-phenoxyacetyl-2'-deoxyadenosine formed was purified by silica gel column chromatography (chloroform-methanol: 100-0/96-4). The desired compound was obtained with a 65% yield (1010 mg). Rf=0.66 in chloroform-methanol 80/20 (v/v).

1H-NMR (PyridineD5): 8.25&9.05(s,2,H2,H8); 7.02(t,H1'); 5.65 (s,2H,CH2 pac); 5.25(m,H3'); 4.6(m,H4'); 4.1-4.35(m,2H,H5'H5''); 2.7-3.3(m,2H,H2'H2''). FAB-MS (PEG matrix, pos.ions): molecular peak +H /e: 386-16% ; N-protected base +H /e: 270-66%.

Preparation of N2-phenoxyacetyl-2'-deoxyguanosine: (compound 3)

2'-deoxyguanosine (4 mmoles; 1070 mg) was dried twice by evaporation from dry pyridine and suspended in 20 ml of dry pyridine. Trimethylchlorosilane (20 mmoles; 2.52 g) was added and mixed at room temperature for 25 minutes. During this lap of time, 1-hydroxybenzotriazole (6.4 mmoles; 0.86 g) was dried three times by evaporation of dry acetonitrile and subsequently suspended in 3 ml of acetonitrile and 3 ml of pyridine. In this second flask was added the phenoxyacetyl chloride (6 mmoles; 0.83 ml). After 5 minutes, both flasks are ice cooled and the flask containing deoxyguanosine was added dropwise to the second flask through a rubber septum. The mixture was stirred overnight at ambient temperature. Tlc prooved that N-acylation was complete, so the mixture was cooled to 5°C and water (2 ml) was added to neutralize any excess of reagents and 2 ml of 29% ammonia were poured in the solution to liberate the two hydroxy functions of the sugar. Complete hydrolysis was checked by tlc. Solvents were removed by rotary evaporation and the subsequent gum was diluted in 75 ml of water. This solution was washed by 3 x 50 ml of chloroform then 3 x 50 ml of diethyl ether. By concentrating the solution, N2-phenoxyacetyl-2'-deoxyguanosine crystallized. After one night at 5°C, the crystals were filtered, rinced with diethyl ether and dried to give 1280 mg (yield=80%). Rf=0.36 in chloroform-methanol 80/20 (v/v).

1H-NMR(pyridine D5): 8.70(s,H8); 6.75(t,H1'); 5.25(s,2H,CH2 pac); 5.1(m,H3'); 4.63(q,H4'); 4.10(m,2H,H5'H5''); 2.7-3.0(m,2H, H2'H2''). FAB-MS(PEG matrix, pos.ions): molecular peak +H /e: 402-13% ; N-protected base +H /e: 286-51%.

Preparation of N2-methoxyacetyl-2'-deoxyguanosine: (compound 4)

2'-deoxyguanosine (4 mmoles; 1070 mg) was dried by two evaporations with pyridine (10 ml), then suspended in 20 ml of dry pyridine and treated with methoxyacetyl chloride (24 mmoles; 2.16 ml). After 3 hours of steering at room temperature, the reaction was checked for completion by tlc. Methanol (3 ml) was added then and the solution was left for 30 minutes. The solvents and the methyl-methoxyacetate formed were evaporated, the following gum diluted in 75 ml of chloroform, and was washed by 2 x 50 ml of 5% NaHCO3 and 50 ml of water. A mixture of triethylamine/ pyridine/water (20/20/60) was added for 15 minutes. The solution was then evaporated to dryness and the desired compound purified by a reverse silica gel chromatography (water-acetone: 100-0/ 70-30). N2-methoxyacetyl-2'-deoxyguanosine was obtained in a 50% yield (690 mg). Rf=0.27 in chloroform-methanol 80/20 (v/v).

1H-NMR (CD3OD): 8.05(s,H8); 6.4(t,H1'); 5.50(m,H3'); 4.33 (m,H4'); 4.15(s,2H,CH2 mac); 3.5(s,3H,CH3 mac); 2.6-3.10(m,2H, H2'H2''). FAB-MS (glycerol matrix, neg.ions): molecular peak -H /e: 338-10% ; N-protected base -H /e: 222-31%.

General method for 4,4'-dimethoxytritylation:

The base-protected nucleoside (4 mmoles) was dried by evaporating 2 x 10 ml of dry pyridine. It was taken up in 20 ml of dry pyridine, ice-cooled, and 4,4'-dimethoxytrityl chloride (4.4 mmoles) was added to the solution. This reaction was left overnight at 5°C and then checked to completion by tlc. Methanol (3 ml) was added and after a couple of minutes, solvents were evaporated to near dryness. The residue was taken up in 100 ml of ethyl acetate, washed by 3 x 75 ml of 5% NaHCO3 and 1 x 75 ml of water. The organic layer was dried over sodium sulfate and evaporated to dryness. The product was then purified on silica gel column with chloroform-methanol.

* 5'-O-(4,4'-dimethoxytrityl)-N4-isobutyryl-2'-deoxycytidine: (compound 5):. Yield=75%. Rf=0.32 in chloroform-methanol 90/10. 1H-NMR (CD3OD): 8.30&7.43(d,2H,H5,H6); 6.15(t,H1'); 4.50(q,H3');

3.7(s,6H,2(CH3) trityl); 1.15(d.d,6H,2(CH3) ibu). FAB-MS (nitrobenzyl alcohol matrix, neg.ions): molecular peak -H /e: 598-34%.

* 5'-O-(4,4'-dimethoxytrityl)-N6-phenoxyacetyl-2'-deoxyadenosine (compound 6):. Yield=60%. Rf=0.50 in chloroform-methanol 90/10. 1H-NMR (CD3OD): 8.57&8.46(s,H2,H8); 6.51(t,H1'); 4.97(s,2H,CH2 pac); 4.68(m,H3'); 3.72(d,6H,2(CH3) trityl). FAB-MS (PEG matrix, pos.ions): molecular peak +H /e: 688-100%.

* 5'-O-(4,4'-dimethoxytrityl)-N2-phenoxyacetyl-2'-deoxyguanosine (compound 7):. Yield=70%. Rf=0.40 in chloroform-methanol 90/10. 1H-NMR (CD3OD) : 8.07(s,H8); 6.45(t,H1'); 5.05(s,2H,CH2 pac); 4.75 (m,H3'); 3.86(s,6H,2(CH3) trityl). FAB-MS(PEG matrix, pos. ions): molecular peak+H /e: 704-15%.

* 5'-O-(4,4'-dimethoxytrityl)-N2-methoxyacetyl-2'-deoxyguanosine (compound 8):. Yield=75%. Rf=0.38 in chloroform-methanol 90/10. H-NMR (CD3OD) : 8.04(s,H8); 6.33(t,H1'); 4.57(m,H3'); 4.12(s,2H, CH2 mac); 3.72(s,6H,2(CH3) trityl); 3.46(s,3H,CH3 mac). FAB-MS (nitrobenzylalcohol matrix, neg.ions): molecular peak -H /e: 640-28%.

General procedure for preparation of phosphotriester derivatives:

Triazole (828 mg; 12 mmoles) was dissolved in 30 ml of dry dioxan. Successively was added at 5°C o.chlorophenyl-phosphorodichloridate (0.98 ml; 6 mmoles) in 10 ml of dioxan, and triethylamine (1.73 ml; 12.5 mmoles) in 10 ml of dioxan. The reaction mixture was shaken for 1 hour at room temperature. Then, in a second flask, dry 5'-O-dimethoxytrityl-N-acylated-nucleoside (4 mmoles) was dissolved in 15 ml of pyridine and added gently into the first flask containing the phosphorylating reagent. The reaction was left for 45 minutes at room temperature until tlc showed completion. Water (3 ml) and triethylamine (3 ml) were added and the subsequent solution stirred 5 minutes. The solvents were evaporated to near dryness and the gum taken up in 75 ml of chloroform, washed by 3 x 50 ml of 5% NaHCO3 and 1 x 50 ml of water. The organic phase was dried on sodium sulfate and evaporated. The nucleotides formed were purified by a silica gel column in dichloromethane and 5-10 % of methanol according to the nature of the nucleic base.

* Isobutyryl-deoxycytidine phosphodiester triethylammonium salt: (compound 9). Yield=70%. 1H-NMR (CD3OD) : 8.19 & 7.56(d,H5,H6);

6.22(t,H1'); 5.20(m,H3'); 1.16(d.d,6H,2(CH3) ibu). 31P-NMR(CD3OD) : -5.3. FAB-MS (glycerol matrix,neg.ions): molecular peak -H /e: 788-2.7%

* Phenoxyacetyl-deoxyadenosine phosphodiester triethylammonium salt:(compound 10). Yield=70%. 1H-NMR (CD3OD): 8.53&8.42(s,H2, H8); 6.56(t,H1'); 5.28(m,H3'); 5.01(s,2H,CH2 pac). 31P-NMR(CD3OD) : -5.5. FAB-MS (glycerol matrix,neg.ions): molecular peak -H /e: 876-6.7%.

* Methoxyacetyl-deoxyguanosine phosphodiester triethylammonium salt:(compound 11). Yield=50%. 1H-NMR (CD3OD): 8.03(s,H8); 6.36 (t,H1'); 5.19 (m,H3'); 4.13(s,2H,CH2 mac); 3.46(s,3H,CH3 mac). 31P-NMR (CD3OD): -5.0. FAB-MS (glycerol matrix, neg.ions): molecular peak -H /e: 830-2.3%.

General procedure for the preparation of the cyanoethyl-diisopropylamine phosphoramidites:

The 5'-O- and N- protected nucleoside (3 mmoles) were dried by coevaporating pyridine and dichloromethane, then dissolved in dry dichloromethane (15 ml). Diisopropylamine (1.5 mmoles) and tetrazole (1.5 mmoles) were added with a syringe through a septum then cyanoethyl-bisdiisopropylamine-phosphine (3.3 mmoles) was added dropwise with a syringe. The reaction was stirred during 35 minutes, tlc showing completion. After filtration of the hydrochloride formed during the reaction, the solvent and the excess of amine were evaporated. The subsequent residue was taken in 100 ml of argon saturated ethyl acetate, washed with 2 x 50 ml of ice-cold 10% sodium carbonate and dried over sodium sulfate. Evaporation of this organic layer gave the desired phosphoramidite derivatives. They were dissolved in toluene (15 ml) for pyrimidines, in ethyl acetate (15 ml) for purines, then precipitated with hexane (250 ml) at -78 °C. Filtration gave white powders. They were further purified by silica gel low pressure chromatography with a hexane-chloroform-triethylamine elution mixture. After evaporation of the solvents, the desired compounds (observed as two diastereoisomers) were dried under reduced pressure in a dessicator, and stored under dry argon.

* Isobutyryl-deoxycytidine phosphoramidite derivative : (compound 12). Yield=85%. 1H-NMR (CD3CN): 8.21&7.12(d.d,H5,H6); 6.14(t,H1') ; 4.60(m,H3'); 1.07(d.d,6H,2(CH3) ibu). 31P-NMR (pyridine D5):

155.2&155.3. FAB-MS (PEG matrix,neg.ions): molecular peak -H /e: 798-21%.

* Phenoxyacetyl-deoxyadenosine phosphoramidite derivative : (compound 13). Yield=90%. 1H-NMR (CD3CN): 8.56&8.28(s,H2,H8); 6.44 (t,H1'); 4.99(s,2H,CH2 pac); 4.93(m,H3'). 31P-NMR (pyridine D5): 155.1&155.3. FAB-MS (PEG matrix,neg.ions): molecular peak -H /e: 886-10.5%.

* Phenoxyacetyl-deoxyguanosine phosphoramidite derivative : (compound 14). Yield=50%. 1H-NMR (CD3CN): 8.12(s,H8); 6.45(t,H1'); 5.05(s,2H,CH2 pac); 4.88(m,H3'). 31P-NMR (pyridine D5): 146,0& 146.2. FAB-MS (PEG matrix, neg.ions): molecular peak -H /e: 903-17%.

General coupling procedure for the assembly of oligonucleotides:

The synthesis were carried out with approximatively 2 µmoles (50 mg) of polymer (Controlled Pore Glass grafted with the 3'-end nucleoside). One cycle of condensation lasting about 10 minutes.

-Detritylation step: 2 minutes of 3% CC13COOH in dichloromethane (or nitromethane).

-Coupling step: In the phosphotriester method, 3 minutes with mesitylene sulfonyl chloride / 1-methyl imidazole as the activator. 2 minutes with tetrazole or nitrophenyltetrazole in the phosphoramidite method.

-Capping: 3 minutes with a mixture of acetic anhydride and dimethylaminopyridine (or 1-methyl imidazole) in acetonitrile.

-Oxidation: Iodine:2.25g (0.45%), 2.2 ml lutidine in 450 ml THF, 50 ml water.

Removal of the oligomer from the CPG-beads, analysis and purification:

Oligomers were treated with 29% NH4OH at 20°C, then the solvents were evaporated and lyophilized. The oligomers were dissolved in 0.5 ml bidistilled water, desalted on a Sephadex G25 column. Each crude solution containing an oligomer was analyzed by 5'-end labelling (32P-ATP and T4 polynucleotide kinase) and polyacrylamide gel electrophoresis. Then they were eluted and sequenced. The oligomers were further purified by H.P.L.C. : the column used was a reverse phase Merck LichroCart RP18-e 4x250 mm, the mobile phase was a gradient of 5 to 13% of pure acetonitrile in 50mM TEAA,(pH=6.6). For shorter oligomers (tetramers), a size-

exclusion gel chromatography "I-125" Waters column was used with 5mM TEAA,(pH=6.6) buffer. Several lyophilizations were needed to eliminate completely the volatile buffer.

Enzymatic digestion of oligomers:

80 µg of chromatographically purified oligonucleotide were taken in 40 µl of water and 10 µl of buffer (2M Tris-HCl,pH=9.0 - 2.5 mM MgCl2). Snake venom phosphodiesterase (1 U), and then alkaline phosphatase (60 U) were added into the Eppendorf tube. Incubation was left for 40 hours at 37°C. The reaction mixture was diluted to 500 µl with water and centrifugated at 12000 g during 15 minutes. The supernatant was transfered in another tube and analyzed by H.P.L.C. with a reverse phase Merck LichroCart, using a gradient of 2 to 40% of acetonitrile in 5 mM TEAA,pH=6.6.

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